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After OVA sensitization and aerosol challenge, SR-AI/II and MARCO-deficient mice exhibited greater eosinophilic airway inflammation and airway hyperresponsiveness compared to wild-type mice. A role for simple SRA-mediated antigen clearance ('scavenging') by lung macrophages was excluded by observation of comparable uptake of fluorescent OVA by wild-type and SRA-deficient lung MΦs and DCs. In contrast, airway instillation of fluorescent antigen revealed significantly higher traffic of labelled DCs to thoracic lymph nodes in SRA-deficient mice than in controls. The increased migration of SRA-deficient DCs was accompanied by enhanced proliferation in thoracic lymph nodes of adoptively transferred OVA-specific T cells after airway OVA challenge. The data identify a novel role for SRAs expressed on lung DCs in down-regulation of specific immune responses to aero-allergens by reduction of DC migration from the site of antigen uptake to the draining lymph nodes.

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INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Our central hypothesis is that lung macrophage scavenger receptors normally function to bind and clear inhaled allergens and pathogens, thereby preventing allergic responses and infections. The purpose of the project is to determine whether 1) decreased levels of SRAs (mediated by environmental stresses) increase susceptibility to asthma or pneumonia; and 2) therapy to increase or maintain normal levels of scavenger receptors will increase resistance to asthma and pneumonia. The scope of the research includes studies using in vivo mouse models (Aim 1), studies of the specific role of alveolar macrophages (Aim 2) and dendritic cells (Aim 3) and studies of the effects of pollutants on scavenger receptors (Aim 4).

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the approved Statement of Work.

For this period, our SOW identified one main task:

Task 1: **Determine susceptibility of SRA 'knockout' (KO) mice to asthma.**
(Months 1-12):

- Measure allergic inflammation (AI) and airway hyperresponsiveness (AHR) after sensitization and challenge with ovalbumin (OVA).
- Compare responses in normal (wild-type) mice, and mice with genetic deletion of SRAI/II, MARCO or both (scavenger receptor knockout mice).
-

We are please to report very good progress in this task which is essentially complete. The work described in this task was summarized in a manuscript for publication which was just recently accepted for publication in the Journal of Immunology (See Appendix 1).

In this work we report that the class A scavenger receptors (SRAs) MARCO and SR-AI/II are expressed on lung macrophages (MΦ) and dendritic cells (DC) and function in innate defenses against inhaled pathogens and particles. Increased expression of SRAs in the lungs of mice in an OVA-asthma model suggested an additional role in modulating responses to inhaled allergen. After OVA sensitization and aerosol challenge, SR-AI/II and MARCO-deficient mice exhibited greater eosinophilic airway inflammation and airway hyperresponsiveness compared to wild-type mice. A role for simple SRA-mediated antigen clearance ('scavenging') by lung macrophages was excluded by observation of comparable uptake of fluorescent OVA by wild-type and SRA-deficient lung MΦs and DCs. In contrast, airway instillation of fluorescent antigen revealed significantly higher traffic of labelled DCs to thoracic lymph nodes in SRA-deficient mice than in controls. The increased migration of SRA-deficient DCs was accompanied by enhanced proliferation in thoracic lymph nodes of adoptively transferred OVA-specific T cells after airway OVA challenge. The data identify a novel role for SRAs expressed on lung DCs in down-regulation of specific immune responses

to aero-allergens by reduction of DC migration from the site of antigen uptake to the draining lymph nodes.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified increased allergic inflammation (AI) and airway hyperresponsiveness (AHR) in SRA-deficient mice in an OVA-asthma model
- Identified SRA expression on dendritic cells as important modulator of allergic response

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

1. Manuscript accepted for publication in J. Immunology (Appendix 1)

CONCLUSION:

We have identified an important role for scavenger receptors in modulating allergic responses using the mouse model. We have made good progress, matching the projected completion of task 1 in year 1. This progress provides a good foundation for moving on to the other tasks in the SOW in the coming year.

REFERENCES: See Appendix 1

APPENDICES:

- 1. Manuscript.

Scavenger receptors SR-AI/II and MARCO limit pulmonary dendritic cell migration and allergic airway inflammation [1]

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Abstract

The class A scavenger receptors (SRAs) [5] MARCO and SR-AI/II are expressed on lung macrophages (MΦ) and dendritic cells (DC) and function in innate defenses against inhaled pathogens and particles. Increased expression of SRAs in the lungs of mice in an OVA-asthma model suggested an additional role in modulating responses to inhaled allergen. After OVA sensitization and aerosol challenge, SR-AI/II and MARCO-deficient mice exhibited greater eosinophilic airway inflammation and airway hyperresponsiveness compared to wild-type mice. A role for simple SRA-mediated antigen clearance ('scavenging') by lung macrophages was excluded by observation of comparable uptake of fluorescent OVA by wild-type and SRA-deficient lung MΦs and DCs. In contrast, airway instillation of fluorescent antigen revealed significantly higher traffic of labelled DCs to thoracic lymph nodes in SRA-deficient mice than in controls. The increased migration of SRA-deficient DCs was accompanied by enhanced proliferation in thoracic lymph nodes of adoptively transferred OVA-specific T cells after airway OVA challenge. The data identify a novel role for SRAs expressed on lung DCs in down-regulation of specific immune responses to aero-allergens by reduction of DC migration from the site of antigen uptake to the draining lymph nodes.

Introduction

The lung is constantly exposed to numerous inhaled particles and pathogens, and relies on the broad ligand specificity of scavenger and other pattern recognition receptors for innate immune defense (1-3). The scavenger receptor family includes two members in the SR-A sub-class which are expressed on lung macrophages and dendritic cells, MARCO (Macrophage receptor with collagenous structure), and SR-AI/II (1, 2, 4). MARCO, like SR-AI/II, binds acetylated LDL and bacteria but not yeast (5-7). MARCO and SR-AI/II expressed on alveolar macrophages function to promote uptake and clearance of inhaled particles and bacteria (7-10).

Aeroallergens constitute another common inhaled challenge to the lung's immune cells. Stimulated in part by gene expression profiling which shows increased expression of MARCO and SR-AI/II in the lungs of mice in an OVA-asthma model, we sought to determine if SRAs contributed to defense of the lung against inhaled allergens using receptor-deficient mice and a model of allergic asthma. We found that sensitized mice lacking SRAs develop more severe airway inflammation (AI) and hyperresponsiveness (AHR) in response to inhaled aeroallergen. Since SRAs mediate macrophage binding and clearance of modified proteins, we initially expected that decreased clearance of antigen (OVA) by SRA-deficient alveolar macrophages (AMs) would be a mechanism for increased allergic responses, but this postulate proved incorrect. We next evaluated the effect of SRA-deficiency on the ability of antigen-loaded pulmonary DCs to migrate to the draining lymph nodes (LNs) and generate specific T cell responses. The data indicate that MARCO and SR-AI/II function in a novel mechanism to down-regulate migration of pulmonary DCs to thoracic lymph nodes, and thereby diminish T cell responses to specific aeroallergens.

Materials and Methods

Animals

Six to 8 weeks old mice genetically deficient in MARCO ($MARCO^{-/-}$) or SR-AI/II ($SR\text{-}AI/II^{-/-}$) were used in all experiments. Age- and sex-matched wild-type (C57BL/6 and BALB/c) mice purchased from Charles River Laboratories (Wilmington, MA) were used as controls.

MARCO$^{-/-}$ mice were generated using targeted homologous recombination (9), and were backcrossed for at least ten generations to the C57BL/6 background. SR-AI/II$^{-/-}$ mice were generated by disrupting exon 4 of the SR-A gene, which is essential for the formation of functional trimeric receptors (11). Double KO mice were obtained in our laboratory by intercross of single knockout mice.

Both single knockouts were backcrossed in our laboratory to the BALB/c background for 8 generations. DO11.10 mice, which are transgenic for the TCR recognizing OVA peptide 323–339 (pOVA$_{323–339}$), A/J (AJ), C3H/HeJ (C3H), and C3H/HeOuJ mice were from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in sterile microisolator cages and had no evidence of spontaneous infection. Approval before all experimentation was obtained from the institutional animal use review committee.

Mouse model of airway allergic inflammation

To compare allergic responses in SRA and normal mice, groups of MARCO$^{-/-}$, SR-AI/II$^{-/-}$ and C57BL/6 WT control mice were sensitized i.p. with 8 μg OVA in 1 mg of alum gel in 0.5 ml of PBS on days 0 and 7. At day 14, the sensitized mice were challenged with aerosolized 0.5 % OVA or PBS for 1 hour. Mice were sacrificed 72 hours post challenge, blood was collected through heart puncture, the lungs were lavaged with PBS before they were harvested , inflated with formalin and processed for histologic analysis.

In BALB/c mice, 10 μg OVA in 2 mg of Alum powder were administered i.p. at days 0 and 7, followed by aerosol challenge with either PBS or 1% OVA for 30 min on days 14 and 15.

Microarray data analysis

Microarray data was acquired from http://pepr.cnmcresearch.org/ (free registration and login required; see project id# 108). Gene expression data were calculated by GeneChip-Robust Multiarray Analysis (GC-RMA) algorithm (12) from bioconductor project (http://www.bioconductor.org/). The fold change in MARCO and SRA gene expression was calculated as the ratio of the level in the OVA/control sample.

The raw p-values were adjusted by false discovery rate correction and an adjusted p-value less than 0.05 was interpreted as significant.

RT and real-time PCR

Total lung RNA was extracted from normal and OVA-sensitized and challenged BALB/c mice using a Qiagen RNAeasy kit according to manufacturer's instructions (Qiagen, Valencia, CA). RNA purity was controlled by optical density measurement. RNA concentrations were adjusted and the samples were reverse transcribed to cDNA using the novel SuperScript® III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). cDNA samples were analyzed in duplicate in a quantitative real-time PCR reaction using the SybrGreen Supermix (Bio-Rad) for MARCO and SRA message using the following primer sequences (IDT, Coralville, IA): SRA, Sense: AGAATTTCAGCATGGCAACTG; Anti-sense: ACGGACTCTGACATGCAGTG; MARCO, Sense: GAAACAAAGGGGACATGGG; Anti-sense: TTCACACCTGCAATCCCTG. Murine b-actin was used as housekeeping control, no-template sample (NTC) was used as a negative control. Data are represented as delta Ct (threshold cycle) values, with lower values indicating greater abundance of mRNA in the sample.

Measurement of airway hyperresponsiveness

AHR was measured in MARCO$^{-/-}$, SR-AI/II$^{-/-}$ and BALB/c WT mice using whole body plethysmography (Buxco; EMKA Technologies, Falls Church, VA) 24h after the last of two daily OVA or PBS aerosol challenges. The response of the airways to inhaled methacholine (Sigma) at concentrations ranging from 6.25 to 100 mg/ml (13) was recorded. AHR was expressed as enhanced pause (P_{enh}), a calculated value that correlates with airway resistance.

Bronchoalveolar lavage

BAL was performed *in situ* with a 20-gauge catheter inserted into the proximal trachea, flushing the lower airways six times with 0.8 ml PBS. The fluid retrieved from the first flushing was kept for ELISA assays. The BAL fluid cells were separated from the BAL fluid by centrifugation, resuspended in PBS, counted, and a fraction was cytospun on microscopic slides for staining with Diff-Quick (Baxter Scientific Products) for subsequent leukocyte differential counts.

Ovalbumin uptake studies

To prepare the OVA-FITC conjugate, OVA was dissolved in carbonate buffer pH 9.2. Freshly prepared FITC in DMSO (10 mg/ml) was added at a ratio of 10 mg per 200 mg of OVA, and the mixture was incubated at RT in the dark for 1 hour. To remove free FITC, the mixture was dialyzed for 24 hours against PBS. MARCO$^{-/-}$, SR-AI/II$^{-/-}$ and C57BL/6 mice were given a 15 min aerosol of a 10 mg/ml solution of OVA-FITC. BALs were performed 1 hour later and cells analyzed by flow cytometry. To test the binding of OVA to AMs *in vitro*, BALF cells ($200x10^3$/well) from C57BL/6 WT and double KO mice were pretreated for 5 min with 5μM Cytochalasin D and then incubated with 5μg/ml OVA-Alexa Fluor 488 for 40 min at 37°C and analyzed by flow cytometry.

Instillation of macromolecule solutions into the trachea

Mice were anesthetized by i.p. injection of 2.5% avertin and received an intratracheal injection (i.t.) of 600 μg OVA-FITC in a volume of 60 μl sterile PBS. The trachea was carefully exposed via a small midline incision and the solution inoculated. The incision was then closed with sterile silk and the mice allowed to fully recover before being returned to the cages.

Preparation of single-cell suspensions and immunofluorescent labeling

Lung digestion medium consisted of RPMI 1640 (from GIBCO BRL) supplemented with 1 mg/ml collagenase type IV (Sigma-Aldrich) and 0.5 mg/ml DNAse (Deoxyribonuclease I from bovine pancreas, Sigma-Aldrich). Lymph node (LN) digestion medium consisted of 1 x Hanks' balanced salt solution (Cellgro®, Mediatech) and 2% EDTA-treated fetal bovine serum (FBS, HyClone) supplemented with 2.5 mg/ml collagenase type IV (Sigma-Aldrich). EDTA-treated FBS was prepared adding 20 μl 0.5 M EDTA per ml of FBS. FACS staining buffer consisted of PBS (free of Ca^{2+} or Mg^{2+}) supplemented with 5% FBS, 0.1% sodium azide and 5mM EDTA.

Preparation of lung and lymph node single-cell suspensions

Lung: Animals were euthanized by CO_2 narcosis. Following thoracotomy, right heart catheterization was performed using a 21G ¾ siliconized needle (Terumo, SURFLO winged

infusion set) and the pulmonary circulation was perfused with at least 20 ml sterile PBS to remove the intravascular pool of cells. 2 ml of digestion medium were then injected in the trachea using a 22G catheter and the trachea was quickly sealed with silk suture after the catheter was removed. The trachea and lungs were then removed, lungs were carefully separated from the heart, thymus and trachea and incubated at 37°C in additional 3 ml of digestion medium for 30 min. Incubation was then prolonged for additional 30 min, with vigorous pipetting of the samples at 10 min intervals with a 5 ml serological pipet. Subsequently, samples were passed through a 70 μm nylon cell strainer, subjected to RBC lysis, incubated in calcium- and magnesium-free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker and finally resuspended in FACS staining buffer and kept on ice until immunofluorescent labeling.

Lymph nodes: For migration studies, animals were euthanized by CO_2 narcosis 24 hours after i.t. injection of OVA-FITC. For T cell proliferation studies animals were euthanized by CO_2 narcosis 96 hours after injection of OVA. Following thoracotomy, paratracheal and parathymic intrathoracic LNs were removed under a stereo microscope (Olympus SZ 60) and incubated at 37°C in 3 ml of LN digestion medium. After 10 min incubation, LNs were minced with 20G 1½ and 25 G 5/8 needles (Becton Dickinson) and incubation was prolonged for another 10 min. Subsequently, samples were passed through a 70 μm nylon cell strainer, incubated in calcium- and magnesium-free PBS containing 10 mM EDTA for 5 min at room temperature on a shaker and finally resuspended in FACS staining buffer and kept on ice until immunofluorescent labeling.

Labeling of single cell suspensions for flow cytometry

All staining procedures were performed at 4°C. Cells were preincubated for 20 min with Fc-receptor blocking antibody (anti-CD16/CD32, BD Biosciences) to reduce nonspecific binding. For lung studies, cells were subsequently stained with PE-Cy5.5 hamster anti-mouse CD11c mAb (Caltag™ Laboratories) and data acquisition was performed using the FL1/FL3 template, to allow assessment of the distribution of CD11c-bright cells with regard to autofluorescence. PE-Cy 5.5 hamster IgG isotype control was used to determine background staining (Caltag™ Laboratories). Rat Anti-F4 / 80 **(**Clone 6F12, IgG2a) and rat anti Mac-3 (clone M3/84, IgG1) were from BD Biosciences Pharmingen. For migration studies, cells were stained with PE-Cy5.5 hamster anti-mouse CD11c mAb (Caltag™ Laboratories). For T cell proliferation studies, cells were stained with PE mouse anti-mouse DO11.10 TCR mAb (clone KJ1-26) (Caltag™ Laboratories). PE-conjugated mouse IgG2a isotype control was used to determine background staining (Caltag™ Laboratories). Flow cytometry data acquisition was performed on a BD FACScan running CELLQuest™ software (Becton Dickinson). FlowJo software (Treestar) was used for data analyis. For lung and migration studies 50,000 total events were acquired for each sample. For T cell proliferation studies 500,000 total events were acquired for each sample. Dead cells were gated out based on light scatter properties.

In vivo assessment of T cell proliferation

CD4(+) T cells were enriched from spleens of DO11.10 mice by magnetic bead separation under sterile conditions using a cocktail of biotin-conjugated monoclonal antibodies against CD8a (Ly-2, rat IgG2a), CD11b (Mac-1, rat IgG2b), CD45R (B220, rat IgG2a), CD49b (DX5, rat IgM) and Ter-119 (rat IgG2b), followed by anti-biotin MicroBeads (colloidal super-paramagnetic MicroBeads conjugated to a monoclonal anti-biotin antibody, clone: Bio3-18E7.2;

mouse IgG1) (Miltenyi Biotec, Auburn, CA). CD4(+) DO11.10 T cells were subsequently labelled with 10 μM 5(6) Carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma) at 37°C for 10 minutes, as described by Lyons (14) and then resuspended in sterile PBS. Mice received an i.v. injection of 10 x 10^6 CFSE-labelled DO11.10 T cells 24 hours before i.t. injection of 600 μg OVA in a volume of 60 μl PBS. 4 days later T cell responses were analyzed in the draining mediastinal LNs by observing CFSE division profiles of live KJ1-26+ CD4+ T cells. The number of transgenic T cells in each LN was calculated as percentage of KJ1-26+/CFSE+ cells among the total cell number.

Statistical Analysis

Student's *t* test (unpaired, two-tailed) was used to calculate significance levels for all measurements. Data are presented as mean ± SEM or SD. Differences were considered significant when $P < .05$.

Results

Increased MARCO and SR-AI/II gene expression in a murine model of asthma

To identify genes modulated in asthma, we analyzed public databases of microarray expression profiling in experimental murine asthma models. Wills-Karp and coworkers determined the response to OVA exposure at 6 and 24 hrs following allergen challenge in both AJ and C3H strains, using 5 replicates of whole lung RNA from each experimental group http://pepr.cnmcresearch.org/. We processed the data as described in Methods. Comparison of allergen-challenged mice to saline-challenged mice revealed significant up-regulation of MARCO and SR-AI/II after exposure to OVA in both strains (Fig. 1A & B). A similar trend was found in studies using the C57BL/6 strain (http://www.ncbi.nlm.nih.gov/projects/geo/gds/gds_browse.cgi?gds=348, GDS348). We also observed increased MARCO and SRA gene expression in RT-PCR analysis of lung samples from OVA-sensitized and exposed mice compared to controls (e. g. dCT values for OVA vs controls: 5.3 ± 0.6 vs. 6.6 ± 0.4 (SRA), 7.2 ± 1.1 vs 9.2 ± 1.5 (MARCO); lower dCT values indicate greater abundance of mRNA target).

Increased severity of airway inflammation in SRA deficient mice during allergic asthma

We next directly analyzed the physiologic relevance of the two SRA receptors, MARCO and SR-AI/II, in vivo in a murine model of allergic airway inflammation caused by OVA sensitization and aerosol challenge. Due to the unavailability of SRA knockout mice in the susceptible A/J and resistant C3H backgrounds used in the microarray studies, we used the C57BL/6 and BALB/c strains, both known to show pulmonary expression of SRAs (8,9) and to be prone to OVA-induced airway inflammation (15,16). Seventy two hours after the aerosol challenge, sham-challenged mice (OVA/PBS groups) showed no sign of inflammation, whereas all OVA/OVA groups showed a remarkable increase in the total number of leukocytes recruited to the airways (Fig. 2 A & B). Notably, the total number of eosinophils and lymphocytes in the BAL samples from OVA/OVA groups was substantially greater in the SRA-deficient mice relative to their control counterparts (Fig. 3 A & B). OVA/PBS mice, in contrast, did not show any recruitment of eosinophils into their airways. Consistent with the increased leukocyte numbers in lavage samples of OVA-challenged SRA-deficient mice, histologic analysis of lungs harvested from these mice showed allergic inflammation consisting of peribronchial and perivascular cell infiltrates of eosinophils and mononuclear cells (Fig. 3 C & D).

Unlike C57BL/6 mice, allergen-sensitized BALB/c mice develop easily detectable AHR following exposure to inhaled allergen (15, 16). MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice on the BALB/c background also showed a significant increase in eosinophils and macrophages (Fig 3E) and total cell number (Fig 2C) in the BALF following OVA challenge, compared to their WT counterparts. The basis for the increased macrophage number in the knockout mice on the BALB/c background is unknown. It is also worth mentioning that the discrepancy in both intensity and nature of cellular inflammatory responses between C57BL/6 and BALB/c strains after exposure to inhaled OVA is an expected result of the different induction protocols we have used to achieve significant eosinophilic recruitment and the antigen dose-dependent response in these strains. Whole body plethysmography was used to evaluate pulmonary function changes after OVA challenge in WT vs MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice. Following aerosolized bronchoconstrictor (methacholine) challenge, WT mice showed a slight, but significant, increase in AHR relative to the baseline ($P<.05$). In contrast, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice showed a much more robust response ($P<0.01$, Fig 3F), consistent with their greater allergic inflammatory response.

WT and SRA-deficient lung MΦs show normal uptake of inhaled OVA allergen

AMs can efficiently bind and internalize unopsonized particles and bacteria through SRAs, leading to the clearance of inhaled matter from the airways and the reduction of the resulting inflammation (7-9), and are known to similarly bind modified proteins (17-19). To determine if SRAs could reduce allergic inflammation by simply 'scavenging' aeroallergen with a resulting decrease in allergen dose, we measured their ability to internalize inhaled allergens using FITC-OVA. WT and KO mice were exposed to inhaled fluorescent OVA, the airways were lavaged 1 hour later and the total fluorescence of AMs was evaluated by flow cytometry. Similar amounts of FITC-OVA were found associated with AMs in WT, MARCO$^{-/-}$and SR-AI/II$^{-/-}$ mice ($P>.05$), indicating essentially identical uptake *in vivo* (Fig 4A). In parallel experiments, FITC-OVA was administered i.t. to the mice and the amount of OVA associated with the MΦ population was determined on the cells isolated from whole lung homogenates. The total amount of FITC-OVA on MΦs, as discriminated by gating of the CD11c(+), F4/80(+) or MAC3(+) populations, was similar in both WT and MARCO$^{-/-}$ mice ($P>.05$, Fig 4B). *In vitro* assays confirmed that the absence of receptors did not affect AM binding of OVA, as double-deficient AMs bound Alexa-OVA to nearly the same extent as did control AMs (data not shown). These findings are consistent with previous reports indicating that, unlike chemically modified albumin, native albumin binding to MΦs is not mediated through SRAs (17-19), and also indicate that nebulization does not per se denature the allergenic proteins sufficiently to create SRA-binding domains.

Allergen-loaded SRA-deficient DCs show increased migration from the lungs to the draining lymph nodes

We next sought to investigate if another SRA-expressing cell type, lung DCs, was involved in the increased asthmatic phenotype seen in SRA-deficient mice. Airway DCs capture antigens in the lungs and migrate to the regional lymph nodes where they present the antigen to the specific T cells. To track DC migration from the lungs to the draining lymph nodes (LN), we administered OVA-FITC intratracheally and analyzed cell suspensions prepared from mediastinal lymph nodes 24 hours later. DCs were labelled with anti-CD11c antibody and the

number of cells expressing the CD11c and also carrying FITC was determined by flow cytometry. While there are no significant differences in lymph node cellularity under basal conditions (Fig. 5A), OVA challenge of the airways resulted in an increase in LN cellularity, an increase which is greater in the SRA-deficient mice (Fig 5B). A striking finding was that SRA-deficient mice showed a significantly greater number of antigen-loaded DCs in the thoracic lymph nodes (Fig 5C), indicating that DC migration is more efficient in the knockout mice. Double KO mice showed an even greater migration of airway DCs after OVA challenge compared to control mice and single deficient mice (Fig. 5D). These studies were performed in unimmunized mice. We next assessed DC migration in OVA-sensitized WT and MARCO$^{-/-}$ mice. Notably, although the sensitized wild-type mice showed an elevated migration of airway DCs to the LNs after OVA exposure (note the expanded range of the Y-axis), the increase was even more marked in the MARCO$^{-/-}$ mice (Fig 5E). To determine if differences in antigen (OVA) uptake by WT or SRA-deficient DCs could mediate the enhanced allergic responses in SRA-deficient mice, we also evaluated the OVA-FITC content of the DCs that reach the LNs after antigen challenge (measured as green fluorescence). We observed the same amounts of antigen in the DCs reaching the LNs in both WT and MARCO$^{-/-}$ mice (Fig 5F). This indicates that SRA-deficiency does not alter uptake of OVA-FITC antigen by DCs, a finding similar to data obtained with macrophages (Fig. 4). To evaluate the potential of trace endotoxin in the ovalbumin preparation to modulate DC migration, we performed OVA-FITC instillation into endotoxin-sensitive and resistant (C3H/Ouj and C3H/HeJ respectively). No differences were observed in the numbers of migrated FITC+ CD11C+ DCs found in thoracic LNs in the two strains of mice (data not shown).

To evaluate the possibility that the enhanced DC migration in KO mice was due to a higher basal number of DCs in the lungs, we quantified the lung DC population in naïve MARCO$^{-/-}$ and control mice. Lung DCs were defined as bright CD11c^{+} cells with low autofluorescence, as described by Vermaelen and Pauwels (20). We found that the number of lung DCs was not statistically different between MARCO$^{-/-}$ and their control WT mice (data not shown).

Allergen challenged SRA deficient mice show enhanced T cell priming in the draining lymph nodes

To more directly test the functional significance of augmented antigen-loaded DC migration in SRA-deficient mice, we used an adoptive transfer model to assess T cell proliferation in the draining LNs after antigen challenge. BALB/c WT, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice were injected i.v. with CFSE-labeled OVA-specific CD4(+) T lymphocytes from DO11.10 transgenic mice. Recipient mice were challenged i.t. with OVA 24 hours later. The mediastinal LNs were harvested 96 hours post-OVA challenge for analysis of dye dilution as a function of cell division.

Comparable numbers of adoptively transferred DO11.10 T cells reached the mediastinal LNs in all 3 groups of mice (data not shown), and similarly, comparable fractions underwent at least one division (% of cells showing decreased CFSE, 91, 93.5, 92 % respectively in WT, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice). However, there was a greater proliferative response in the lymph nodes of MARCO$^{-/-}$ mice ($295 \pm 83 \times 10^3$, Mean ± SD) compared to wild-type mice ($140 \pm 83 \times 10^3$), indicating that a higher absolute number of T cells had undergone a greater number of divisions in the MARCO$^{-/-}$ mice (Fig 6A, B & C). This indicates that the larger numbers of

antigen-loaded DCs that migrate to the draining LNs of the MARCO$^{-/-}$ mice result in a greater proliferative response by antigen-specific T lymphocytes. SR-AI/II$^{-/-}$ mice showed a similar trend which did not reach statistical significance in T cell proliferation ($206 \pm 92 \times 10^3$) compared to control mice.

Discussion

The data presented identify a novel role for SRAs expressed on lung dendritic cells in modulating pulmonary responses to aeroallergens. The context for our findings includes the recognition of the important role of dendritic cells (DC) in the pathogenesis of asthma (21), and as professional antigen-presenting cells which bridge innate and adaptive immunity (22, 23). DCs express SR-AI/II, which functions in antigen presentation and adaptive immunity (17, 19, 24-28). For example, SR-AI/II$^{-/-}$ mice are deficient in mounting an efficient T cell response to maleylated murine serum albumin, a known SR-AI/II ligand (29). In contrast, the role of MARCO receptors in modulating adaptive immunity has not been examined.

It has been postulated that MARCO expression is induced upon DC maturation (30, 31). Although we did not directly address the maturation state of pulmonary DCs in naive WT mice, we know that 1) only immature DCs can take up and process antigen (32), 2) immunohistochemical studies show expression of MARCO only on macrophages in the normal lung (8), with absence of MARCO-labeling in normal airways that contain CD11c+ airway dendritic cells, and 3) mediastinal LN DCs express MARCO after OVA challenge (data not shown). This suggests that pulmonary DCs start to express MARCO after allergen encounter, consistent with the increased MARCO gene expression observed after OVA challenge in microarray studies.

Some limitations of the study merit discussion. For some control experiments, only MARCO-deficient mice were analyzed (e.g., migration of DCs in OVA-sensitized mice, Figure 5E). Hence, the full extent to which SR-AI/II deficiency mirrors the findings with MARCO deficient mice requires further characterization. One potential problem to be considered is confounding effects of trace endotoxin in the OVA allergen. Two lines of evidence argue against this possibility. First, no differences were observed in the numbers of migrated FITC+ CD11C+ DCs found in thoracic LNs endotoxin-sensitive and resistant (C3H/Ouj and C3H/HeJ respectively). Second, we have previously reported similar levels of cytokine release (TNF-alpha and MIP-2) by AMs from WT and KO mice in response to LPS in vitro (9), arguing against differential responses on this basis.

In peripheral tissues, such as the lungs, DCs exist normally in an immature state and provide a sentinel function for foreign antigens (32). Upon antigen encounter, DCs undergo a process of maturation, which triggers their migration to draining lymph nodes and enhances their antigen-presenting capacity (23). The migration of antigen-loaded DCs from peripheral tissues to the lymph nodes is a critical step in generating an optimal immune response (33, 34), and hence a potential regulatory point.

SRAs may inhibit DC migration through a number of mechanisms. SRAs have been shown to promote adhesion to matrix molecules (35, 36) and to other cells, e.g., marginal zone macrophages to B cells (37), and either of these interactions could potentially reduce cell migration. Pikkarainen et al. (38) have previously shown that fibroblastic cell lines transfected with MARCO undergo significant morphologic changes through induction of dendritic plasma membrane processes. These processes include the appearance of large lamellipodia-like structures and long plasma membrane extensions. Moreover, a clear correlation exists between

MARCO expression and the rearranged actin cytoskeleton of mature DCs (30), although in this study MARCO expression upon maturation was associated with a decrease in filopodia and a round phenotype. The morphologic changes induced by simple MARCO expression do not require its interaction with any given ligand. The rearrangements induced by MARCO in fibroblastic cell lines were shown to be partially dependent on Rac1 (38). Rac, together with Rho and Cdc42, represent a group of small GTPases involved in the formation of filopodia and podosomes in immature DCs (39), structural changes that could increase adhesion and reduce migration. To test the speculation that adhesion to matrix might mediate some of the observed effects, studies of the kinetics of induction of MARCO expression on airway and lymph node dendritic cells after allergen challenge are warranted, as well as comparison of the migration capacity of MARCO deficient and wild-type DCs. Additional mechanisms are suggested by data indicating the ability of scavenger receptors, when present, to skew the cytokine milieu and immune response toward Th1 type immunity (40). Indeed, one of the mechanisms leading to increased AHR in allergic asthma is enhanced recruitment of eosinophils into the allergen-challenged airways, as we observed in the lungs of SRA deficient mice. This may be a consequence of increased recruitment of Th lymphocytes and an altered cytokine milieu.

Innate immune responses are increasingly recognized as critical modifiers of adaptive immunity (41, 42). In the example presented here, the innate pattern recognition receptors, the SRAs, mediate reduced amounts of total antigen delivery to lymph nodes (through decreased numbers of DCs carrying similar amounts of antigen per cell). SRA-mediated down-regulation of lung immune responses likely contributes to reduction of unwanted immune responses to commonly encountered environmental aeroallergens.

Figure legends

Figure 1. Augmented expression of MARCO and SR-AI/II in allergic asthma. Microarray analysis of RNA transcripts for MARCO (A) and SR-AI/II (B) was performed on asthmatic and control A/J and C3H mice at 6 h and 24 h post-allergen or PBS challenge. Data are expressed as Mean ± SD of normalized gene expression obtained from 5 mice. *P<.05 vs. same time point after PBS challenge.

Figure 2. BAL cell yields after OVA-sensitization and challenge. Control (C57BL/6 or BALB/c), MARCO$^{-/-}$and SR-AI/II$^{-/-}$ mice were sensitized twice with OVA in Alum and exposed once to 1% aerosolized OVA (+OVA). As a negative control, mice were immunized with OVA and challenged with PBS aerosol (+PBS). Seventy two hours post-aerosol exposure, lungs were lavaged and then fixed in formalin for H&E staining. Total leukocyte counts were determined in BALF of MARCO$^{-/-}$, SR-AI/II$^{-/-}$ and their wild type counterparts in both C57BL/6 (A and B) and BALB/c (C) backgrounds. Data shown here are representative of 18 mice (A), 12 mice (B), and 6 mice per group (C). **P<.01 and ***P<.001; for OVA vs. PBS challenge.

Figure 3. Response to OVA challenge in wild-type vs. SRA-deficient mice. Differential counts showing the amounts of BALF macrophages, eosinophils and lymphocytes were determined on stained cytospin slides from MARCO$^{-/-}$, SR-AI/II$^{-/-}$ and their wild type counterparts in both C57BL/6 (A and B) and BALB/c backgrounds (E). Data shown here are representative of 18 mice per group (A), 12 mice per group (B), and 6 mice per group (E). *P<.05, **P<.01 and

***P<.001; for OVA vs. PBS challenge. Representative photomicrographs of tissue histopathology are shown from WT (**C & D**), MARCO$^{-/-}$ (**C**) and SR-AI/II$^{-/-}$ (**D**) mice, challenged with either PBS or OVA. Inset (C) shows higher magnification (600X) of eosinophils and mononuclear cells comprising the inflammatory infiltrates. Images are shown at a 200X magnification for MARCO$^{-/-}$ and 100X for SR-AI/II$^{-/-}$ mice. SRA-deficient mice also show enhanced airway hyperreactivity (F), measured by whole body plethysmography in conscious OVA-sensitized and challenged BALB/c mice. P_{enh}, an index of airway obstruction, was recorded after aerosolization of either PBS or 50mg/ml Methacholine. Data represents mean Penh values ± SEM (*n* = 6 mice/group for OVA/OVA and 3 mice/group for OVA/PBS). # P<.05 vs. PBS challenge, **P<.01 vs. WT mice.

Figure 4. Absence of SRAs on AMs does not affect the clearance of inhaled allergen.
Control (C57BL/6), MARCO$^{-/-}$and SR-AI/II$^{-/-}$ mice were challenged with aerosolized OVA-FITC (10mg/ml) for 15 min. The lungs were lavaged 1 hour later with PBS and cells were analyzed by flow cytometry to compare uptake of fluorescent OVA (**A**). Data shown represent the mean ± SEM of 4 mice per group. In separate experiments (**B**), 3 wild-type and 3 MARCO$^{-/-}$ mice received 600 μg of OVA-FITC intratracheally. The lungs were harvested 4 hours later and cell suspensions prepared by homogenization were labeled with antibodies to either CD11c, F4/80 or MAC3 antigens and mean green fluorescence intensity of the double positive cells was determined.

Figure 5. Increased migration of MARCO$^{-/-}$and SR-AI/II$^{-/-}$ pulmonary DCs in response to an inhaled antigen challenge. Total cell content of homogenized mediastinal lymph nodes from C57BL/6, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice was determined before (**A**) or 24 hours after (**B**) they received 600 μg of OVA-FITC intratracheally. LN suspensions from OVA-FITC challenged mice were stained for CD11c and the fraction of FITC^{+} DCs was determined by flow cytometry (**C**). A similar protocol was applied to double knockout mice (**D**) and OVA-sensitized C57BL/6 and MARCO$^{-/-}$ mice (**E**). Also, the amount of green fluorescence associated with the DCs was determined (**F**). Data represent the Mean ± SD from 2 or more separate experiments with ≥ 6 mice per genotype, * p< .05; ** p< .01.

Figure 6. Enhanced antigen-inducedT lymphocyte proliferation in mediastinal lymph nodes of SRA-deficient mice. CFSE-labeled spleen CD4^{+} T cells from DO11.10 mice were transferred i.v. into BALB/c, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice 24 hours prior to i.t. administration of OVA. Ninety six hours later, cell suspensions were prepared from draining lymph nodes and stained with KJ-126-PE antibody. Representative histograms (**A,** cells in the rectangles have undergone at least one division) and dot plots (**B;** Cells in the M2 zone have undergone at least one division) are shown for control, MARCO$^{-/-}$ and SR-AI/II$^{-/-}$ mice groups. The deduced absolute number of cells that underwent at least one division is shown in (**C**). Data represent the Mean ± SD from 8 (MARCO$^{-/-}$) and 12 mice (SR-AI/II$^{-/-}$). * *P*< .05.

Footnotes:

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[5] Abbreviations used in this paper: AHR, airway hyperresponsiveness; AI, airway inflammation; AM, alveolar macrophage; BAL(F), bronchoalveolar lavage (fluid); CCL, CC chemokine ligand; DC, dendritic cell; LN, lymph node; LPS, lipopolysaccharides; MARCO, macrophage receptor with collagenous structure; MFI, mean fluorescence intensity; MΦ, macrophage; OVA, ovalbumin; SR, scavenger receptor; SR-AI/II, scavenger receptor A type I and II.

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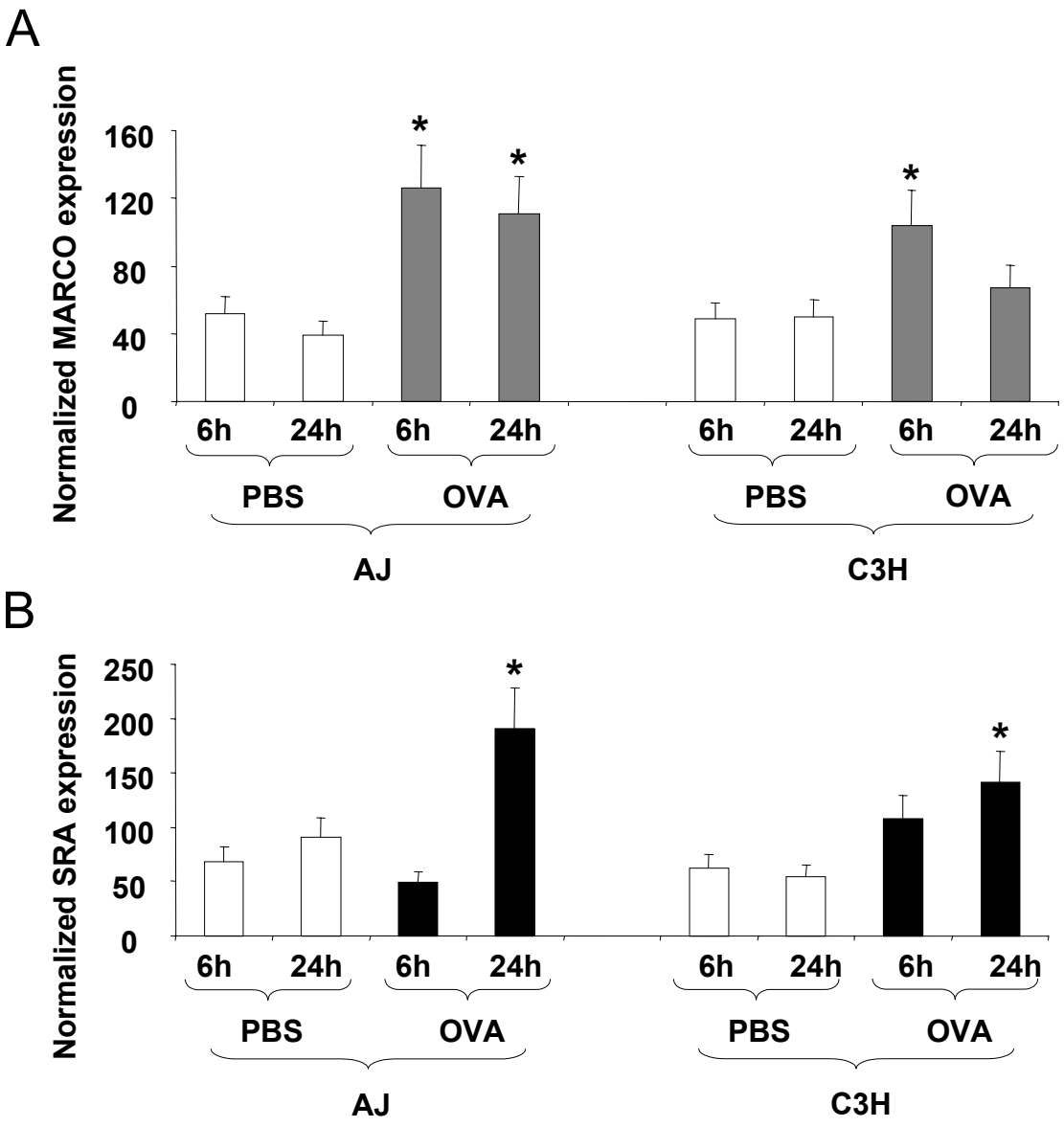
Fig.1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B
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100
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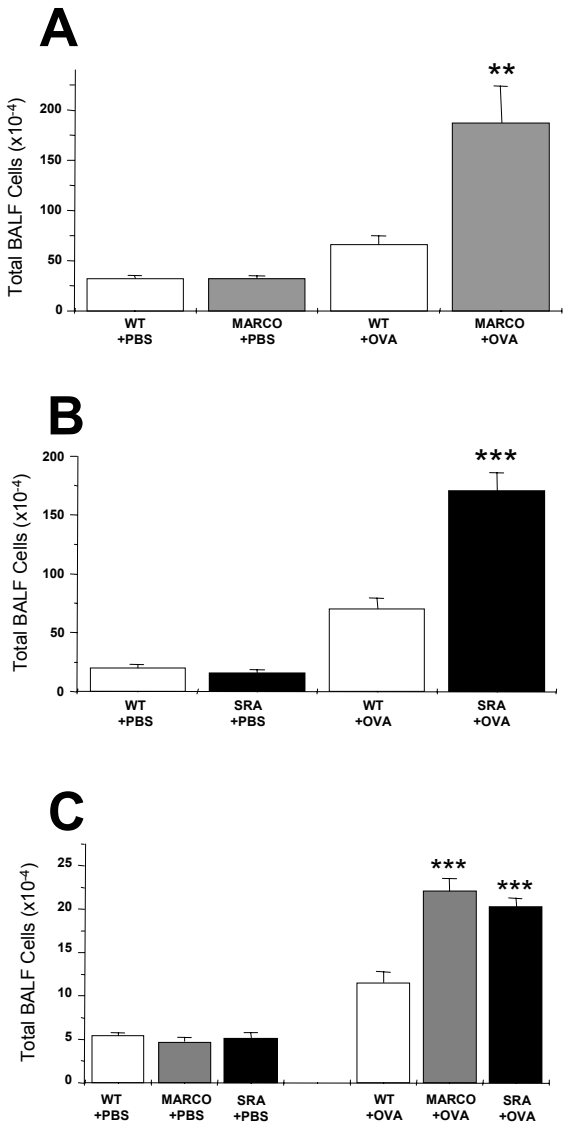
Fig.2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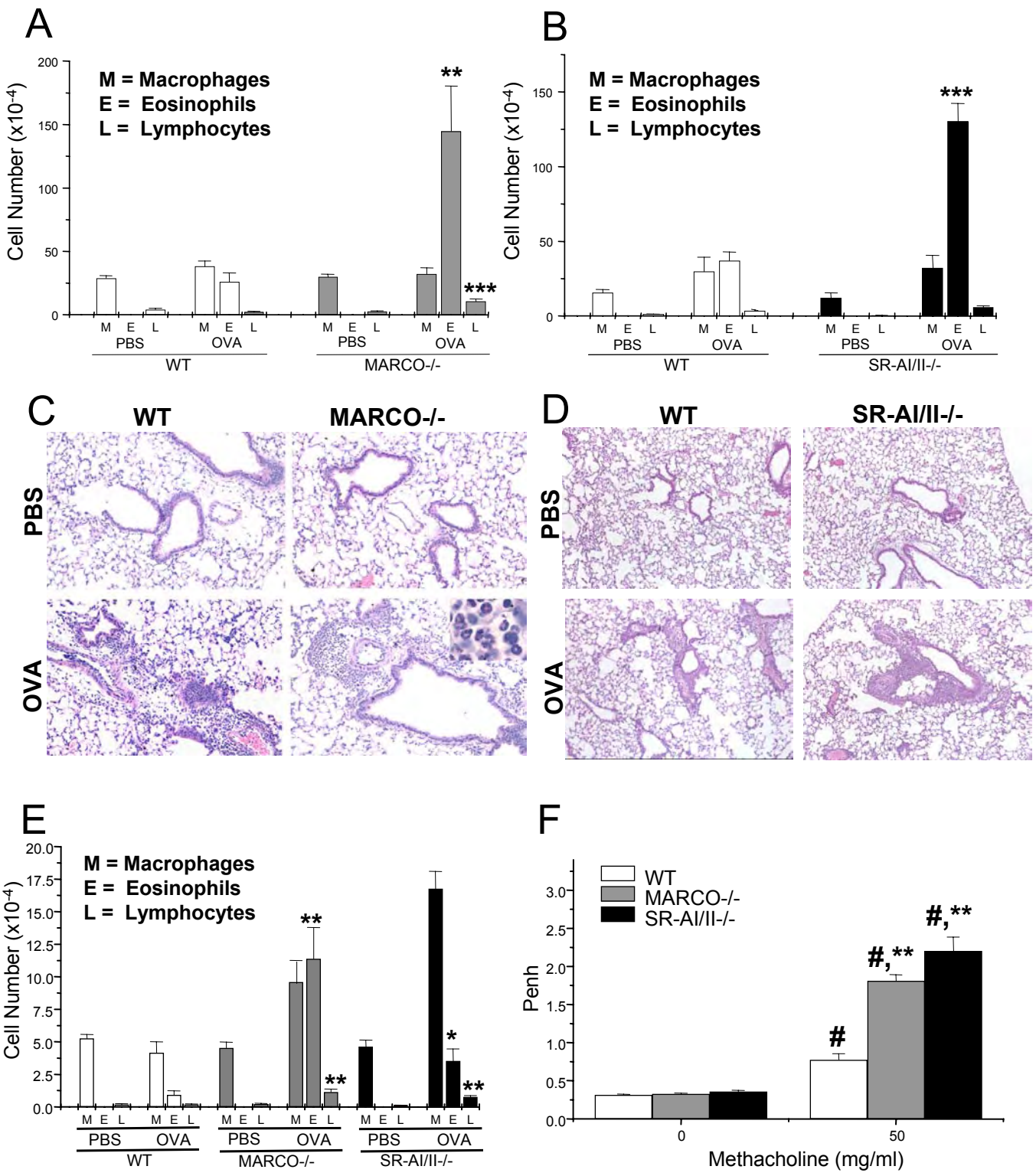
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M = Macrophages
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L = Lymphocytes
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MARCO-/-
SR-AI/II-/-
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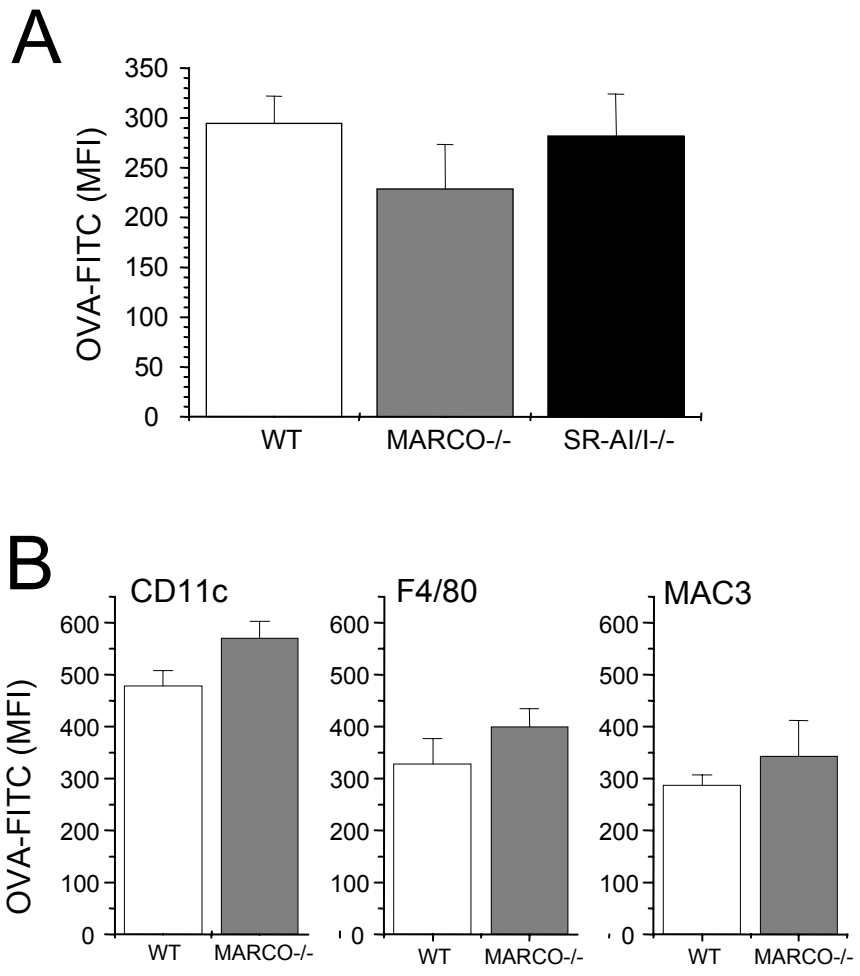
Methacholine (mg/ml)

Fig.4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SR-AI/I-/-
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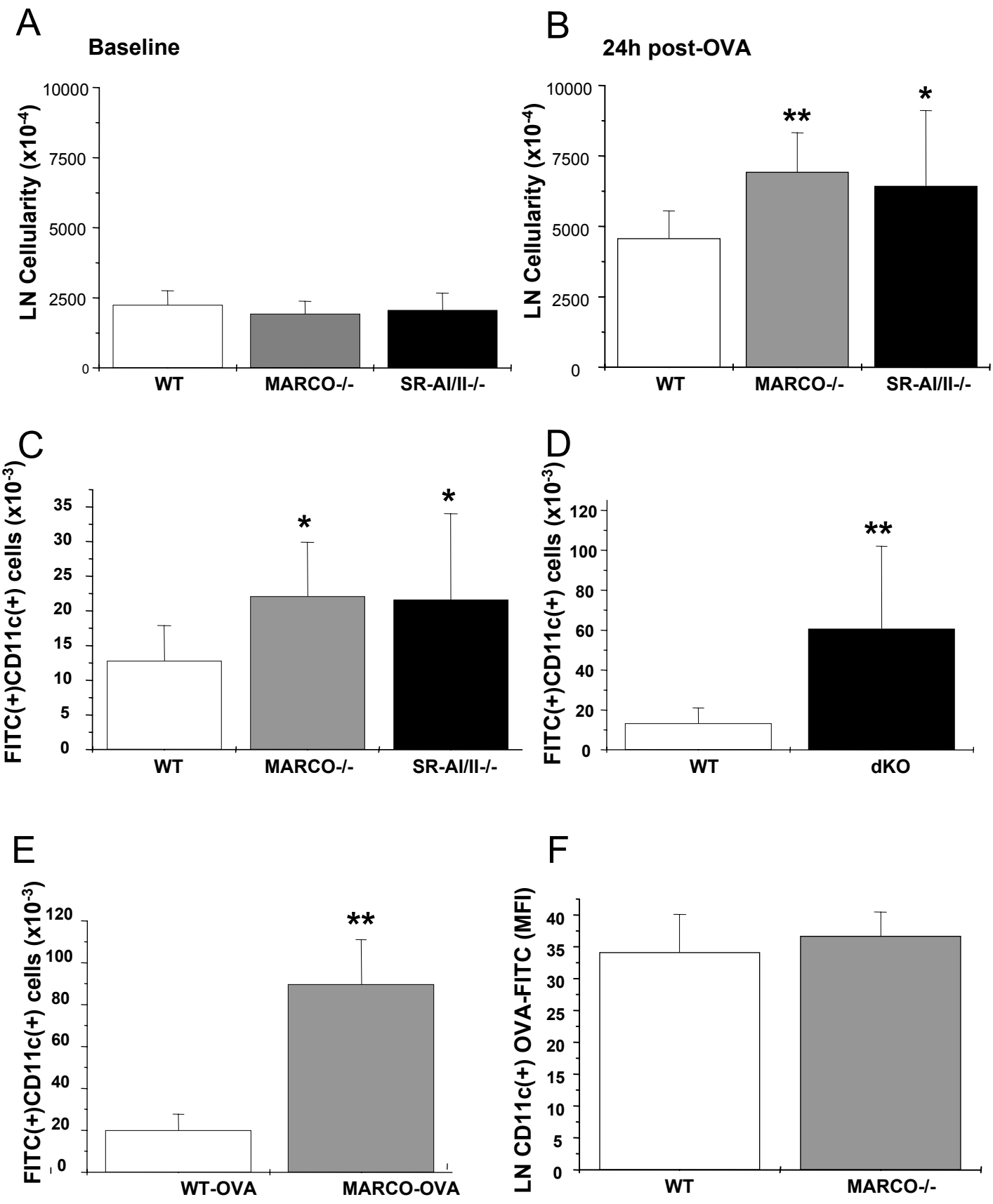
Fig.5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**
*
C
FITC(+)CD11c(+) cells (x10-3)
35
30
25
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15
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D
FITC(+)CD11c(+) cells (x10-3)
120
100
80
60
40
20
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dKO
E
FITC(+)CD11c(+) cells (x10-3)
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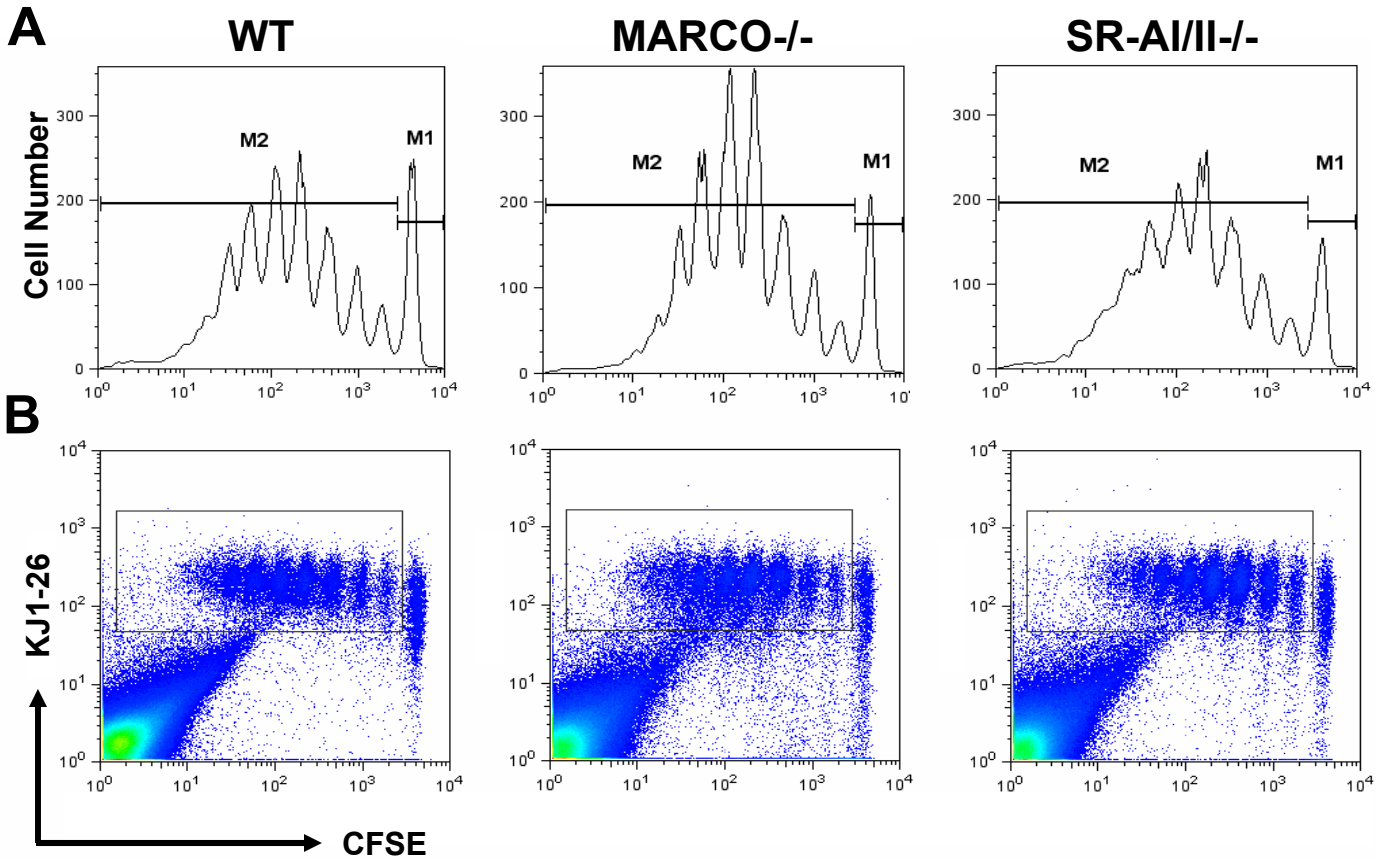
Fig.6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SR-AI/II-/-
Cell Number
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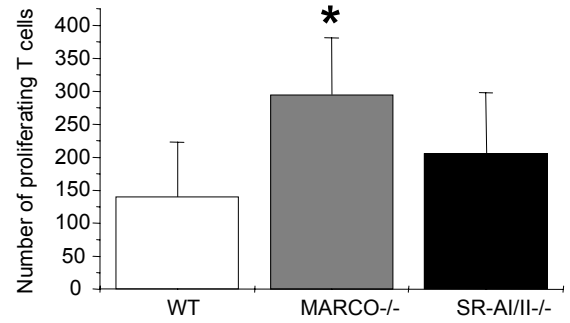
M1
B
KJ1-26
CFSE


C
Number of proliferating T cells
*
WT
MARCO-/-
SR-AI/II-/-